(54) Title: BACTERIAL NITROREDUCTASE FOR THE REDUCTION OF CB 1954 AND ANALOGUES THEREOF TO A CYTOTOXIC FORM

(57) Abstract

The invention provides a nitroreductase, obtainable from a bacterium having the following characteristics as exemplified by examples isolated from *Escherichia coli B* and *Bacillus amyloliquifaciens*: 1) it is flavoprotein having a molecular weight in therange 20-60 Kilodaltons; 2) it requires either NADH or NAD(P)H or analogues thereof as a cofactor; 3) it has a Km for NADH or NAD(P)H in the range 1-100 µM; and 4) it is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form e.g. the hydroxylamine. The sequence of one such nitroreductase is shown in Seq. ID No. 1. The nitroreductase may be conjugated to a tumour targeting agent such as a monoclonal antibody and used to convert prodrugs into active antitumour agents. Such prodrugs and drugs are also provided.

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BACTERIAL NITROREDUCTASE FOR THE REDUCTION OF CB 1954 AND ANALOGUES THEREOF TO A CYTOTOXIC FORM

THIS INVENTION relates to the control of neoplastic tissue growth and is particularly concerned with the provision of new anti-tumour agents and with enzymes capable of converting 5 prodrugs into anti-tumour agents.

The alkylating agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (hereinafter designated CB 1954) has been known, almost for 20 years, as an interesting experimental compound of unique selectivity. Although CB 1954 is structurally quite closely 10 related to numerous other known alkylating agents which have a relatively broad range of activity, CB 1954 exhibits considerable activity against the Walker tumour cells in vivo or in vitro but was thought to be virtually inactive against other tumours.

15 It was recently discovered that the selectivity of CB 1954 arose from the fact that it was not an anti-tumour agent per se but was a prodrug for an anti-tumour agent generated from CB 1954 by a nitroreductase enzyme found in the Walker cell. This nitroreductase from the Walker cell was subsequently shown to 20 be an enzyme known from other sources which was an NAD(P)H dehydrogenase (quinone) classified as EC.1.6.99.2, see Robertson et al, J. Biol. Chem. 261, 15794-15799 (1986).

In the course of the previous investigations with CB 1954, it was found that the Walker cell enzyme EC.1.6.99.2 had the 25 ability to reduce the 4-nitro group of CB 1954 to the corresponding hydroxylamine and that it was the resulting 5-(aziridin-1-yl)-2-nitro-4-hydroxylamino-benzamide that was the active anti-tumour agent.

The use of prodrugs represents a clinically very valuable 30 concept in cancer therapy since, particularly where the prodrug is to be converted to an anti-tumour agent under the influence of an enzyme that is linkable to a monoclonal antibody that will bind to a tumour associated antigen, the combination of such a prodrug with such an enzyme monoclonal/antibody

conjugate represents a very powerful clinical agent.

We have now discovered new nitroreductases, obtainable from
bacterial sources, that are of interest in that not only are
they capable of converting CB 1954 into an active anti-tumour
5 agent, but also, unlike EC.1.6.99.2, capable of converting CB
1954 analogues which are also prodrugs into active anti-tumour
agents.

Description of the Drawings

Figure 1 shows the results of an experiment in which CB 1954
10 (100μM) and reduced cofactor (500μM) were incubated with enzyme
(2mg/ml) E. coli nitroreductase (A) or 25μg/ml Walker DT
diaphorase (B) in 10mM sodium phosphate buffer (pH7) in air at
37°C. At various times aliquots (10μl) were injected onto a
Partisil SCX (240 x 4.7mm) HPLC column and eluted isocratically
15 (2ml/min) with 100mM NaH_2PO_4. The eluate was continuously
monitored for absorption at 320, 260 and 360 nm and the
concentration of CB 1954 calculated by integration of the peak
corresponding to this compound on the HPLC trace.
Figure 2 shows the formation of actinomycin D (AMD) during
20 incubation of an AMD prodrug with a nitroreductase of the
present invention.
Figure 3 shows the formation of mitomycin C (MC) during
incubation of an MC prodrug with a nitroreductase of the
present invention.
25 Figure 4 shows the binding in vitro of an antibody-enzyme
conjugate according to the invention to cells.

The present invention provides a nitroreductase, obtainable
from a bacterium having the following characteristics as
exemplified by examples isolated from Escherichia coli B and
30 Bacillus amyloliquifaciens:

1.   It is a flavoprotein having a molecular weight in the
range 20-60 Kilodaltons;

2.   It requires either NADH or NAD(P)H or analogues thereof as

a cofactor.

3.   It has a Km for NADH or NAD(P)H in the range
1-100μM.

4.   It is capable of reducing either or both nitro groups of
5 CB 1954 and analogues thereof to a cytotoxic form e.g. the
hydroxylamine.

The nitroreductases of the invention occur naturally within the
cells of E. coli B , E. coli C and other E. coli strains e.g.
K12 type as well as other gram negative organisms e.g. Thermus
10 aquaticus, and gram positive bacteria such as Bacillus
amyloliquifaciens and Bacillus caldotenax. They can be
recovered from such cells by disrupting the cells and
subjecting the cell contents to chromatographic separation and
isolating the nitroreductase.

15 For example, the nitroreductase of the present invention from
E. coli B has been purified to homogeneity − see Table 1 and
has been subjected to amino acid sequence analysis with the
results set out in Table 2.   The upper sequence shows the
deduced amino acid sequence of the 219-mer nitroreductase
20 obtained from Salmonella typhimurium as described by Watanabe
et al, Nucl. Acids, Res. 18, 1059 (1990). The lower sequence
in bold type shows the sequence of the cyanogen bromide
fragments of the E. coli B nitroreductase as an example of the
present invention showing a certain degree of homogeneity but
25 sufficient differences to confirm that it is nitroreductase
that is different from that of Watanabe et al and the recently
described Enterobacter cloacae nitroreductases, see Bryant et
al, J. Biol Chem. 266, 4126 (1991) or the Walker cell
nitroreductase and is a previously unreported enzyme.

30 The amino acid sequence of the E. coli B nitroreductase of the
invention can also been derived from sequencing the nucleotides
in the nitroreductase gene and these sequences are set out
below in Table 3. The nucleotide sequence of Table 3 has been
used to prepare the attached sequence listings.

Using the information in Table 3, a nitroreductase according to
the present invention may be prepared by expressing DNA
encoding the nitroreductase in a suitable expression vector
contained in a host cell, and recovering the nitroreductase.

5 The expression vector may be, for example, a bacterial, yeast,
insect or mammalian expression vector and the host cell will be
selected to be compatible with the vector.

As indicated above, the new enzymes of the present invention
are capable of reducing a nitro group in various substrate
10 molecules and we have found that the enzymes are particularly
useful in their ability to reduce the nitro group of various p-
nitrobenzyloxycarbonyl derivatives of cytotoxic compounds to
give "self-immolative" compounds that automatically decompose
to release cytotoxic compounds.

15 The interest in the present approach resides in the fact that
the cytotoxicity of various cytotoxic compounds containing
amino or hydroxy substituents, particularly aromatic amino or
hydroxy substituents give rise to p-nitrobenzyloxycarbonyl
derivatives of the amino or hydroxy group which exhibit
20 considerably less cytotoxicity than the amino or hydroxy parent
compound. Thus, it is possible to use the p-nitro-
benzyloxycarbonyl derivatives as prodrugs in a system of the
type discussed above where the prodrug is converted into an
anti-tumour agent under the influence of an enzyme that is
25 linkable to a monoclonal antibody that will bind to the tumour
associated antigen.

Accordingly, the present invention provides new compounds of
the general formula:
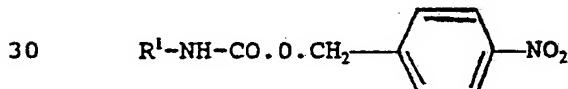
30 $\quad$ R^1-NH-CO.O.CH$_2$—⟨benzene ring⟩—NO$_2$ $\qquad$ I

and:

$$R^2\text{-O.CO.O.CH}_2\text{---}\langle\text{phenyl}\rangle\text{---NO}_2 \qquad \text{II}$$

where R^1 and R^2 are groups such that the compound R^1NH_2 and R^2OH are cytotoxic compounds.

It is preferred that compounds R^1NH_2 and R^2OH are aromatic cytotoxic compounds and the compounds R^1NH_2 can be any one of the well known nitrogen mustard compounds, for example based on p-phenylene diamine. Thus, the compound R^1NH_2 can be:

$$(ClCH_2CH_2)_2N\text{---}\langle\text{phenyl}\rangle\text{---NH}_2 \qquad \text{III}$$
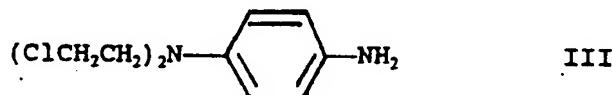
or analogues of this compound with the general structure IV

$$(ClCH_2CH_2)_2N\text{---}\langle\text{phenyl}(R', R'')\rangle\text{---NH}_2 \qquad \text{IV}$$
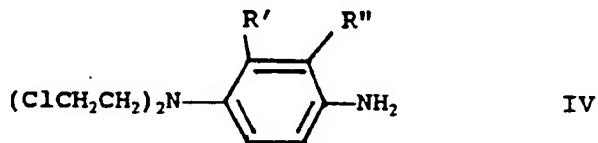
where R' and R'' are H, F or CH_3, and particularly where

     $R' = H$ and $R'' = CH_3$;

or $R' = CH_3$ and $R'' = H$;

or $R' = H$ and $R'' = F$;

or $R' = F$ and $R'' = H$.

A further type of amino cytotoxic compound that can be used in accordance with the present invention are compounds such as actinomycin D, doxorubicin, daunomycin and mitomycin C. The structure of the pro-drugs derived from actinomycin D, doxorubicin and mitomycin C are shown below as V, VI and VII respectively.

MeVal — Sar — Pro — D-Val — (O) — Thr — CO

MeVal — Sar — Pro — D-Val — Thr — (O) — CO — NHCOOCH$_2$ — NO$_2$

N

O

CH$_3$

CH$_3$

V

O

OH

COCH$_2$OH

OH

CH$_3$O

O

OH

O

H$_3$C

O

HO

NH

COOCH$_2$ — NO$_2$

VI

O

NH$_2$

CH$_3$

O

N

CH$_2$OCONH$_2$

OCH$_3$

NCOOCH$_2$ — NO$_2$

VII
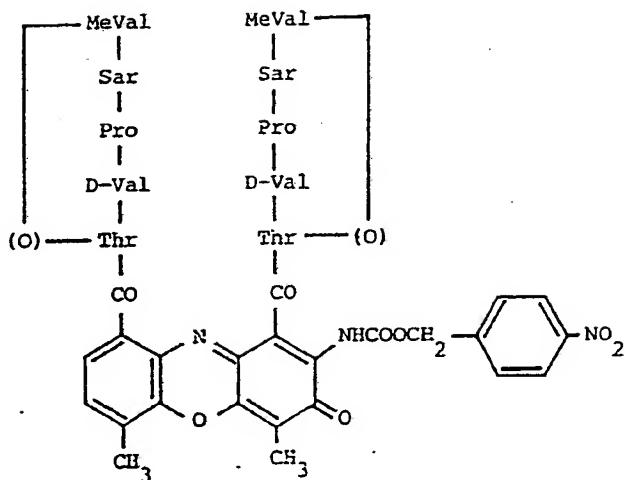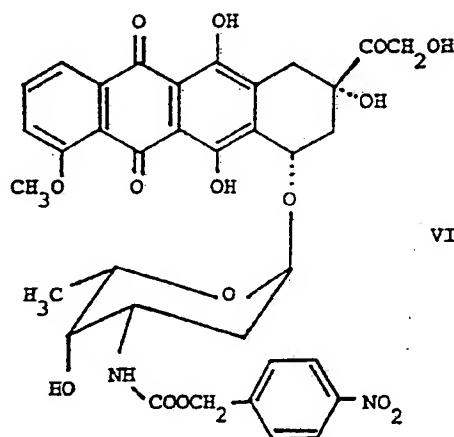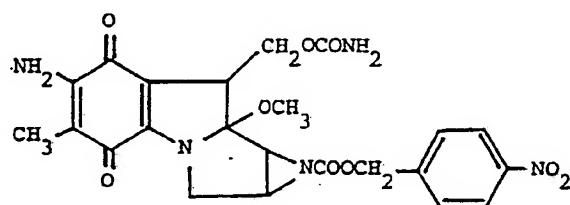
SUBSTITUTE SHEET

Similar p-nitrobenzyloxy derivatives can be made at the amino
substituent of other actinomycins and of the other cytotoxic
compounds of the type mentioned above.

In addition to forming p-nitrobenzyloxycarbonyl derivatives at
5 an amino group on a cytotoxic compound, similar derivatives can
be made at a hydroxy group, particularly a phenolic hydroxy
group of a cytotoxic compound. Here, attention is directed at
the phenolic nitrogen mustard compounds, and a specific
compound of the invention of this type is of the formula:

10
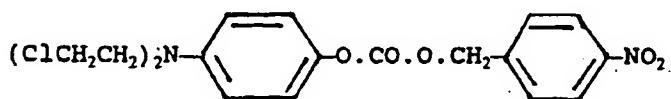
$$(ClCH_2CH_2)_2N-\!\!\left\langle\!\bigcirc\!\right\rangle\!\!-O.CO.O.CH_2\!\!\left\langle\!\bigcirc\!\right\rangle\!\!-NO_2 \qquad\qquad VIII$$

In a further aspect of the invention, we have determined that
the new enzymes of the present invention are capable not only
15 of reducing at least one of the nitro groups of CB 1954 but
also at least one of the nitro groups of certain CB 1954
analogues, e.g. descarboxamido CB1954 (1-aziridin-1-yl-2,4-
dinitrobenzamide - known as CB 1837) and N,N-dimethyl CB 1954
[N,N-dimethyl-(5-aziridin-1-yl-2,4-dinitrobenzamide also known
20 as CB 10-107]. The new enzymes of the present invention are
also capable of reducing the nitro groups of other aromatic
nitro compounds such as 5-chloro-2,4-dinitrobenzamide, 3,5-
dinitrobenzamide, 3-nitrobenzamide, 4-nitrobenzamide and 5-
nitro-2-furaldehydesemicarbazone (nitrofurazone).

25 As indicated above, the nitroreductases of the present
invention are capable of converting CB 1954 into
2-hydroxylamino-4-nitro-5-(aziridin-1-yl)benzamide which is an
active anti-tumour agent and the present invention extends to
such 2-hydroxylamino compounds together with its acylated and
30 etherified derivatives. That is to say, the present invention

provides compounds of the general formula IX:

     IX
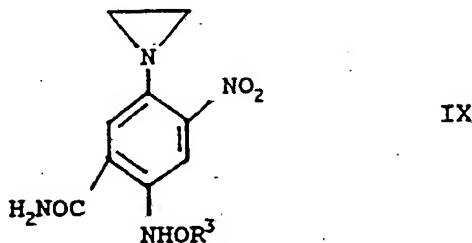
wherein R^3 is H, an acyl group or a hydrocarbyl group
containing up to 6 carbon atoms. By acyl group, we mean a
carboxylic acyl group R^4CO where R^4 is a hydrocarbyl group
containing 1 to 6 carbon atoms, e.g. an alkyl, alkenyl or
phenyl group. The hydrocarbyl group R^3 can be a C_{1-6} alkyl,
alkenyl or phenyl group.

The present invention also provides hydroxylamino derivatives
of the general formula X:
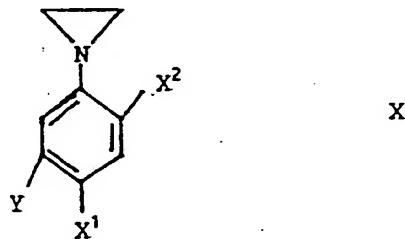
     X

wherein X^1 and X^2, which may be the same or different, are each
$NHOR^5$ or NO_2 with the proviso that X^1 and X^2 are not both NO_2,
where R^5 is H or a carboxylic acyl or hydrocarbyl group as
defined above in relation to formula IX and Y is H or
$CON(CH_3)_2$. In the compounds of formula X containing two
hydroxylamino groups, the group R can be the same or different
but will normally be the same.

As mentioned above, the new p-nitrophenylbenzyloxy compounds of
the invention of formulae I and II are of interest in that they
have a reduced cytotoxicity compared to that of the cytotoxic
compound R^1NH_2 or R^2OH from which they are derived and they are

capable of acting as a substrate for the nitroreductase of the
present invention. While the present invention is not
dependent, for its definition, upon the exact mode of action of
the nitroreductase on the compound of formula I or II, it is
5 believed that the nitro group of the p-nitrophenyl-benzyloxy-
carbonyl residue is converted to the corresponding amino or
hydroxylamino group and that the resulting p-aminobenzyloxy-
carbonyl or p-hydroxyl-aminobenzyloxycarbonyl compound
automatically degrades under the reaction conditions used for
10 the enzymatic reduction to release the cytotoxic compound and
form p-aminobenzyl alcohol or p-hydroxylaminobenzyl alcohol
and carbon dioxide as by products in accordance with the
following reaction scheme:

15   R^1-NH-CO.O.CH_2——⟨benzene ring⟩——NO_2 __nitroreductase__ →

          R^1-NH-CO.O.CH_2——⟨benzene ring⟩——NH_2 ——————————————→

20
          R^1NH_2 + CO_2 +   HO.CH_2——⟨benzene ring⟩——NO_2
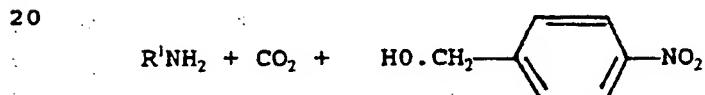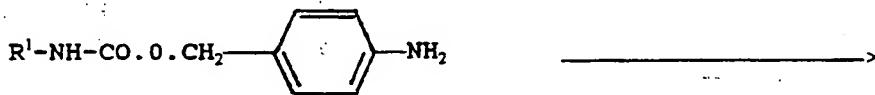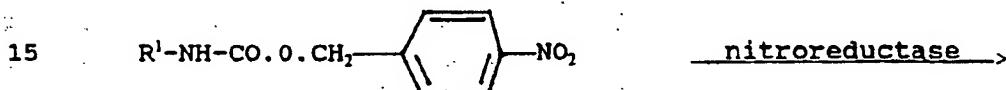
The p-nitrobenzyloxycarbonyl compounds of the invention are
conveniently prepared by methods of chemical synthesis known
25 per se. For example, the amine or hydroxy cytotoxic compounds
can be reacted with 4-nitrobenzyl chloroformate under anhydrous
conditions in the presence of a hydrogen chloride aceptor,
particularly an alkylamine such as triethylamine. This
reaction can be carried out in a dry organic solvent such as
30 chloroform and the resulting compound of the invention of
formula I or formula II isolated from the organic solvent by
conventional methods such as chromatography.

The new compounds of the present invention IX and X will
normally be prepared by subjecting the corresponding dinitro
35 compound to the action of the new nitroreductase of the present

invention.   It is, of course, also possible to produce the new
compounds of the present invention IX and X by chemical
synthesis, using selective reducing agents followed by
conversion of the resulting hydroxylamine to its corresponding
5 ester or ether.   The esters and ethers are more conveniently
prepared semi-synthetically by carrying out the reduction of
the nitro group with the nitroreductase of the present
invention to give the corresponding hydroxylamine that is then
converted by known chemical methods to the corresponding ester
10 or ether.

One of the most convenient ways of producing the new
nitroreductase of the invention is to recover the material from
the cell contents of bacteria such as E. coli B.
Alternatively, it is possible to clone the gene, isolatable
15 from the bacteria, encoding the desired enzyme and to transfer
the cloned gene, in a suitable vector system, into another host
cell from which or in which it can be expressed.

In order to bring about the enzymatic reduction of CB 1954 and
its analogues with the new enzymes of the present invention, it
20 is necessary to have a cofactor present in the reaction system.
The ability of the enzyme of the present invention to bring
about this reduction can be demonstrated experimentally by the
use of NADH or NAD(P)H as the cofactor but the use of such
cofactors in clinical practice may be problematic in view of
25 the ease with which NAD(P)H particularly is oxidised by other
enzymes present in the body and the lack of selectivity of the
cofactors between the various mammalian and non-mammalian
enzymes.   We have now found that the riboside of 1,4-dihydro-
nicotinic acid is as least as effective as a cofactor in the
30 nitroreductase reduction of CB 1954 and analogues thereof and
moreover, because of its selectivity to the E. coli
nitroreductase of the invention, is more suited to clinical use
which makes its incorporation in a multi-component system of
the type described below particularly valuable.

35 The riboside of 1,4-dihydro-nicotinic acid which can be used in
the present invention as a cofactor is a new compound and forms

part of the present invention.   It can be prepared from
commercially available nicotinic acid ribotide which is first
converted to the corresponding riboside by enzymatic
dephosphorylation e.g. using an alkaline phosphatase.   The
5   riboside, obtained by such enzymatic dephosphorylation, or by
chemical synthesis, using the method described by Jarman, J.
Chem. Soc. (c) 918-920 (1969) can then be reduced, e.g. using
an alkali metal hydrosulphite, to give 1,4-dihydro-nicotinic
acid riboside.

10 One of the most important practical applications of the new
enzymes of the present invention is that they can be used in
association with nitro compounds that are prodrugs for anti-
tumour agents and so provide a system of cancer chemotherapy
where the extent of exposure of the patient to the cytotoxic
15 agent is limited, so far as possible, to those regions where
there is the interaction between the prodrug and the
nitroreductase of the invention.   Thus, one aspect of the
present invention is to provide a method of chemotherapy and a
system for chemotherapy involving the conjoint use of the
20 nitroreductase of the present invention in association with a
nitro compound which is a prodrug for a cytotoxic compound.

The most or one of the most convenient ways of utilising the
system of the present invention is to conjugate the
nitroreductase of the present invention to a targeting agent
25 such as a monoclonal antibody that will bind with a tumour-
associated antigen.

As used herein, the term "monoclonal antibody" will be
understood by those of skill in the art not simply to refer to
antibodies produced by traditional hybridoma techniques, but
30 also to cover antibodies and variants thereof produced by
recombinant means.   These include, for example, humanised
antibodies such as those with a constant region from a human
antibody grafted onto a non-human antibody variable region (see
for example EP-A-O 120 694), chimeric antibodies such as those
35 with non-human complementarity determining regions (CDRs)
grafted into a human variable region framework (see for example

EP-A-0 239 400) and single chain antibodies. Fragments of such
monoclonal antibodies which retain their target binding
activity are also included by the general term "monoclonal
antibody". This includes Fab' and F(ab')$_2$ fragments.

5 The selection of monoclonal antibody will clearly be influenced
   by the nature of the target tumour but for the purposes of
   illustrating the present invention, reference may be made to
   the anti-CEA antibody A$_5$B$_7$.

   As an alternative to the use of a monoclonal antibody, it is
10 also envisaged that other targeting agents to which the
   nitroreductase of the present invention is conjugated may be
   used. For example, it is known that certain soluble
   macromolecules can be used for passive tumour targeting of
   certain tumour types. Many solid tumours possess vasculature
15 that is hyperpermeable to macromolecules. Although the reasons
   for this are not clearly understood, the result is that such
   tumours can selectively accumulate circulating macromolecules.
   The enhanced permeability and retention effect (EPR effect) is
   thought to constitute the mechanism of action of SMANCS
20 (styrene/maleic-anhydride-neocarzinostatin), now in regular
   clinical use in Japan for the treatment of hepatoma. Another
   class of conjugates under investigation for anticancer activity
   is N-(2-hydroxypropyl)methacrylamide copolymer-anthracycline
   conjugates (L. Seymour, Critical Reviews in Therapeutic Drug
25 Carrier Systems, 9(2) 135-187 (1992)). Thus, conjugates of a
   polymer, including styrene/maleic-anhydride or N-(2-hydroxy-
   propyl)methacrylamide copolymer, and the nitroreductase of the
   invention can be used place of conjugates of a monoclonal
   antibody and enzyme.

30 With this system, it is possible in a course of cancer
   chemotherapy to administer to the patient requiring the
   treatment the nitro compound which is the prodrug for the
   cytotoxic compound and the enzyme/targeting agent conjugate.
   The prodrug and the conjugate can be administered
35 simultaneously but it is often found preferable, in clinical

practice, to administer the enzyme/agent conjugate before the
prodrug, e.g. up to 72 hours before, in order to give the
enzyme/agent conjugate an opportunity to localise in the region
of the tumour target.   By operating in this way, when the
5 prodrug is administered, conversion of the prodrug to the
cytotoxic agent tends to be confined to the regions where the
enzyme/agent conjugate is localised, i.e. the region of the
target tumour and damage to healthy cells caused by the
premature release of the cytotoxic agent is minimised.

10 The degree of localisation of the enzyme/agent conjugate (in
terms of the ratio of localized to freely circulating active
conjugate) can be further enhanced using the clearance and/or
inactivation systems described in WO89/10140. This involves,
usually following administration of the conjugate and before
15 administration of the prodrug, the administration of a
component (a "second component") which is able to bind to the
such part of the conjugate so as to inactivate the enzyme
and/or accelerate the clearance of the conjugate from the
blood. Such a component may include an antibody to the
20 nitroreductase of the invention which is capable of
inactivating the enzyme.

The second component may be linked to a macromolecule such as
dextran, a liposome, albumin, macroglobulin or a blood group O
erythrocyte so that the second component is restrained from
25 leaving the vascular compartment. In addition or as an
alternative, the second component may include a sufficient
number of covalently bound galactose residues, or residues of
other sugars such as lactose or mannose, so that it can bind
the conjugate in plasma but be removed together with the
30 conjugate from plasma by receptors for galactose or other
sugars in the liver. The second component should be
administered and designed for use such that it will not, to any
appreciable extent, enter the extravascular space of the tumour
where it could inactivate localised conjugate prior to and
35 during administration of the prodrug.

The exact dosage regime will, of course, need to be determined
by individual clinicians for individual patients and this, in
turn, will be controlled by the exact nature of the prodrug and
the cytotoxic agent to be released from the prodrug but some
5 general guidance can be given.   Chemotherapy of this type will
normally involve parenteral administration of both the prodrug
and the enzyme/agent conjugate and administration by the
intravenous route is frequently found to be the most practical.

Bearing in mind the manner in which the enzymes of the present
10 invention are to be used, the present invention extends to
pharmaceutical compositions comprising the enzyme, preferably
conjugated to a targeting agent such as a monoclonal antibody
capable of binding to a tumour-associated antigen in
association with a pharmaceutically acceptable carrier or
15 diluent, normally one suitable for parenteral administration.

The present invention also extends to pharmaceutical
compositions comprising one or more of the p-nitro-
benzyloxycarbonyl compounds of the present invention of formula
I, formula II, formula V, formula VI or formula VII in
20 association with a pharmaceutically acceptable carrier or
diluent, normally one for parenteral administration.

The present invention further extends to pharmaceutical
compositions comprising the hydroxylamino anti-tumour agents of
the present invention of formula IX or formula X in association
25 with a pharmaceutically acceptable carrier or diluent, normally
one for parenteral administration.

The present invention also provides a system for use in the
control of neoplasia in a human or animal subject comprising
the nitroreductases of the present invention, preferably
30 conjugated with a targeting agent such as monoclonal antibody
that will bind to a tumour-associated antigen, in association
with at least one of a p-nitrobenzyloxycarbonyl compound of
Formula I, II, V, VI or VII which is a prodrug for an anti-
tumour agent and preferably a riboside or ribotide of nicotinic
35 acid or nicotinamide to act as a cofactor for the enzyme.   The

present invention extends to a method of treating neoplasia in
a human or animal host requring such treatment which comprises
administering to the host an effective amount of a p-
nitrobenzyloxycarbonyl compound of Formula I, II, V, VI or VII
5 which is a prodrug for an anti-tumour agent and the enzyme of
the present invention, preferably conjugated with a targeting
agent such as a monoclonal antibody that will bind to a tumour-
associated antigen, the enzyme preferably being used in
association with an ribotide or riboside of nicotinic acid or
10 nicotinamide as cofactor for the enzyme.

The present invention further provides a system for use in the
control of neoplasia in a human or animal subject comprising a
nitroreductase of the present invention, preferably conjugated
with a targeting agent such as a monoclonal antibody that will
15 bind to a tumour-associated antigen, in association with a
nitro compound which is a prodrug for an anti-tumour agents of
the formula IX or X and preferably a riboside or ribotide of
nicotinic acid or nicotinamide to act as a cofactor for the
enzyme.   The present invention also provides a method of
20 treating neoplasia in a human or animal host requiring such
treatment which comprises administering to the host an
effective amount of a nitro compound which is a prodrug for an
anti-tumour agents of the formula IX or X and the enzyme of the
present invention, preferably conjugated with a targeting agent
25 such as a monoclonal antibody that will bind to a tumour-
associated antigen, the enzyme preferably being used in
association with an ribotide or riboside of nicotinic acid or
nicotinamide as cofactor for the enzyme.

The various systems for use in the treatment of neoplasia
30 described above optionally include the "second component" for
accelerated clearance described above. Likewise, the methods
of treatment of neoplasia described above optionally include as
part of that method the use of the second component, an
effective amount of which is administered after administration
35 of the enzyme, in order to increase the ratio of localised to
freely circulating enzyme. Reference may be made to WO89/10140
for further particular details of the second component, and

such details can be incorportated for use in the present invention.

The present invention is further illustrated by the following Examples.

EXAMPLE I

Isolation and purification of a nitroreductase enzyme
from E. coli B.

200 grams of E. coli B cell paste were resuspended to a total
5 volume of 1 litre of 20mM potassium phosphate buffer, pH 7,
containing 0.3M ammonium sulphate. The cells were broken
ultrasonically using an MSE Soniprep 150 disintegrator (3 x 30
seconds on full power with 60 second intervals to allow heat to
dissipate). To aid clarification of the extract, DNase (23,000
10 Kunitz units/L) and RNase (2,400 Kunitz units/L) were added
prior to centrifugation at 8,000g for 30 minutes to remove cell
debris. The clear yellowish supernatant was passed through a
0.45μm filter prior to chromatography.

The filtered extract was applied to a column
15 (25 x 5 cm) of Phenyl-Sepharose CL-6B (Pharmacia) in 20mM
potassium phosphate buffer, pH 7, containing 0.3M ammonium
sulphate. After washing with 2 column volumes of starting
buffer, the column was eluted with 10mM Tris-HCl buffer, pH
7.6. Active fractions were pooled and dialysed for 18 hours
20 against 20mM Tris-HCl, pH 7.6, to remove traces of ammonium
sulphate. The dialysed fractions were applied in 50 ml
aliquots to Q-Sepharose-High Performance in 20mM Tris-HCl, pH
7.6, at a flow rate of 4 ml per minute. Elution was by a 0-
0.2M gradient of KCl, the nitroreductase eluting at 0.1-0.12M
25 KCl. Active fractions were pooled and desalted into 20mM Bis
Tris propane, pH 7, using a column (32 x 6 cm) of Sephadex G25
medium. These fractions were applied to Q-Sepharose High
Performance (Hi-Load 26/10 column, Pharmacia) equilibrated in
20mM Bis Tris propane, pH7. Elution was by a 0-0.1M gradient
30 of KCl. Nitroreductase eluted as the first major peak at 0.07-
0.09M KCl.

Homogeneity of the final product was ascertained using precast
8-25% gradient gels for native polyacrylamide gel
electrophoresis (Pharmacia Phastsystem). Electrophoresis was
35 performed for 75vh.

The nitroreductase in crude and partially purified fractions was routinely assayed by its quinone reductase activity using menadione as substrate, NADH as co-factor and cytochrome C as terminal electron acceptor.

5 Determination of Isoelectric Point

The isoelectric point of nitroreductase was determined by isoelectric focusing (Pharmacia Phastsystem, focusing for 400 vh) and chromatofocusing using a Mono P column (Pharmacia Mono P HR5/20, 20mM Bis-Tris pH 6.3 and polybuffer 74, pH 4.0).

10 The E. coli nitroreductase was isolated as a pure protein with a molecular weight of 24kDa (as determined by both SDS-polyacrylamide gel electrophoresis and gel filtration chromatography). A second protein, which had quinone reductase activity but was inactive as a nitroreductase against

15 CB 1954, partially co-elutes from Phenyl Sepharose and can be fully separated from the active enzyme by the ion exchange chromatography step on Q-Sepharose high performance (see Table 1) at pH 7.6. The two enzymes differ in molecular weight (inactive 55KDa; active 24KDa) and isoelectric point (inactive

20 5.2; active 5.1). The active protein has a yellow coloration suggesting the presence of a flavin coenzyme. After heating at 70°C for 20 minutes this flavin could be separated from the apoenzyme by ultrafiltration and shown to be FMN, rather than FAD, using HPLC.

TABLE 1

The purification of a nitroreductase from E. coli B. The enzyme activity was assayed, at 37°C, by its quinone reductase activity using menadione (10μM) as substrate, NADH (500μM) as cofactor and cytochrome C (70μM) as terminal electron acceptor. A unit was defined as 1μmole of cytochrome C reduced per minute.

FRACTION	TOTAL ACTIVITY (Units)		SPECIFIC ACTIVITY (Units/mg protein)	YIELD (%)	
	CB 1954 ACTIVE	CB1954 INACTIVE		CB 1954 ACTIVE	CB 1954 INACTIVE
Crude	3784*		0.34	100	
Phenyl Sepharose	2371*		1.6	63	
	1109	1262		30	33
Q-Sepharose (Tris, pH7.6)	666	-	79	18	-
Q-Sepharose (Bis-Tris propane, PH7)	310	-	130	8	-

*This includes activity from enzymes not active against CB 1954

Fragments suitable for sequence analysis were produced by digestion of the enzyme with cyanogen bromide. The peptides which resulted from these digests were purified by reverse-phase HPLC, using a RP 300 column (25 x 4.6 mm) (Brownlee) and
5 a solvent gradient of 10-60% acetonitrile in water with 0.06% trifluoroacetic aid in each solvent. Sequence analysis was performed by automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer (Kelvin Close, Warrington, U.K.).

10 <u>Amino acid sequence analysis of the nitroreductase</u>

The <u>E. coli</u> nitroreductase as isolated above was subject to amino acid sequence analysis. In contrast to the enzyme isolated from the Walker tumour, EC.1.6.99.2, the nitroreductase of the present invention was not blocked at the
15 N-terminus and gave a clear N-terminal sequence of 31 amino acid residues and a peptide, generated by digestion with cyanogen bromide, of a further 41 residues. These partial sequences are given in Table 2.

TABLE 2

Amino acid sequences of the N-terminus of the E. coli B nitroreductase
and of a peptide obtained after digestion of the nitroreductase with
cyanogen bromide (bold type) and a comparison with the deduced protein
sequence of the nitroreductase from Salmonella typhimurium (Watanabe
et al, 1990).

```
1                     10                    20                    30
M D I V S V A L Q R Y S T K A F D P S K K L T  A  E  E  A  D  K I K T L
M D I I S V A L K R H S T K A F D A S K - L T (P) E  Q  A (D) Q I K

                 40                    50                    60
L Q Y S P S S T N S Q P W H F I V A S T E E G K A R V A K S A A G N Y

   70                    80                    90                   100
T F N E R K M L D A S H V V V F C A K T A N D D A W L E R V V D Q E D

                110                   120                   130
A D G R F A T P E A K A A N D K G R R F F A D M H R V S L K D D H Q W

      140                   150                   160                   170
M  A K V V Y L N V G N F L L G V A A M G L D A V P I E G F D A E V L
(M) A K Q V Y L N V G N F L L G V A A L G L D A V P I E G F D A A I L

         180                   190                   200
D A E F G L K E K G Y T S L V V V P V G H H S V E D F N A G L P K S R
D A E F G L K I

210                   219
L P L E T T L T E V
```

Nucleotide sequence of the nitroreductase gene
The nitroreductase gene of E. coli has been cloned and its nucleotide
sequence determined by dideoxy sequence analysis of both strands. In
Table 3 is shown the nucleotide sequence of 1167 base NruI/Pst
fragment which contains an open reading frame of 651 nucleotides
encoding the nitroreductase. Putative sequence of Shine-Delgarno and
transcriptional termination signal are indicated.

- 22 -

TABLE 3

<u>NruI</u>
TCGCGATCTGATCAACGATTCGTGGAATCTGGTGGTTGATGGTCTGGCTAAACGCGATCA   60

AAAAAGAGTGCGTCCAGGCTAAAGCGGAAATCTATAGCGCATTTTTCTCGCTTACCATTT   120
                                             <u>S.D.</u>
CTCGTTGAACCTTGTAATCTGCTGGCACGCAAAATTACTTTCACA<u>TGGAGT</u>CTTTATGGA   180
                                                        m   d

TATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACT   240
  i   i   s   v   a   l   k   r   h   s   t   k   a   f   d   a   s   k   k   l

TACCCCGGAACAGGCCGAGCAGATCAAAACGCTACTGCAATACAGCCCATCCAGCACCAA   300
  t   p   e   q   a   e   q   i   k   t   l   l   g   y   s   p   s   s   t   n

CTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGTGTTGCCAA   360
  s   q   p   w   h   f   i   v   a   s   t   e   e   g   k   a   r   v   a   k

ATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGT   420
  s   a   a   g   n   y   v   f   n   e   r   k   m   l   d   a   s   h   v   v

GGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGGCTGAAGCTGGTTGTTGACCAGGA   480
  v   f   c   a   k   t   a   m   d   d   v   w   l   k   l   v   v   d   q   e

AGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGATAAAGGTCGCAA   540
  d   a   d   g   r   f   a   t   p   e   a   k   a   a   n   d   k   g   r   k
                      <u>BglII</u>
GTTCTTCGCTGATATGCACCGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACA   600
  f   f   a   d   m   h   r   k   d   l   h   d   d   a   e   w   m   a   k   q

GGTTTATCTCAACGTCGGTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGGT   660
  v   y   l   n   v   g   n   f   l   l   g   v   a   a   l   g   l   d   a   v

ACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGG   720
  p   i   e   g   f   d   a   a   i   l   d   a   e   f   g   l   k   e   g

CTACACCAGTCTGGTGGTTGTTCCGGTAGGTCATCACAGCGTTGAAGATTTTAACGCTAC   780
  y   t   s   l   v   v   v   p   v   g   h   h   s   v   e   d   f   n   a   t

GCTGCCGAAATCTCGTCTGCCGCAAAACATCACCTTAACCGAAGTGTAATTCTCTCTTGC   840
  l   p   k   s   r   l   p   q   n   i   t   l   t   e   v   .
    <u>terminator</u>
CGGGCATCTGCCCGGCTATTTCCTCTCAGATTCTCCTGATTTGCATAACCCTGTTTCAGC   900

CGTCATCATAGGCTGCTGTTGTATAAAGGAGACGTTATGCAGGATTTAATATCCCAGGTT   960

GAAGATTTAGCGGGTATTGAGATCGATCACACCACCTCGATGGTGATGATTTTCGGTATT   1020

ATTTTTCTGACCGCCGTCGTGGTGCATATTATTTTGCATTGGGTGGTACTGCGGACCTTC   1080

GAAAAACGTGCCATCGCCAGTTCACGGCTTTGGTTGCAAATCATTACCCAGAATAAACTC   1140
    <u>PstI</u>
TTCCACCGTTTAGCTTTTACCCTGCAG   1167

EXAMPLE 2

Enzymatic reduction of CB 1954. CB 1954 (100μM and also
containing [U-³H] CB 1954 at 1.6 x 10⁵ dpm per nmole), NADH or
NAD(P)H (500 μM) were incubated with the active enzyme obtained
5   as described in Example 1, (generally 2μg/ml E. coli
nitroreductase or 35μg/ml Walker NAD(P)H dehydrogenase
(quinone) EC 1.6.99.2) in 10 mM sodium phosphate buffer
(pH 7) under either air or helium. At various times aliquots
(10μl) were injected onto a Partisphere SCX (110 x 4.7 mm) HPLC
10   column and eluted isocratically (2 ml/min) with 100mM NaH₂PO₄.

The eluate was continuously monitored for adsorption at 310,
260 and 360 nm and the spectra of eluting components recorded
using a diode-array detector. Samples (0.5 ml) were collected
and the tritium activity of each determined by liquid
15   scintillation counting. This separation system could resolve
all the expected reduction products.

To confirm further the identity of any reduction products, the
above reduction mixture was also injected onto an ODS-5 reverse
phase HPLC column and eluted (1 ml/min) with a methanol
20   gradient (0-30% linear over 30 min, 30-100% linear over 10
min.) in 0.1 M sodium phosphate buffer (pH 7).

Reduction of CB 1954 by the E. coli nitroreductase resulted in
the formation of two products, shown to be, by comparison of
retention times and spectral characteristics with known
25   standards, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide
and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide. No
other CB 1954 metabolites were found. In further confirmation
of the formation of both the 2 and the 4-hydroxylamines by the
nitroreductase of the invention, 33μM of 4-hydroxylamine was
30   formed when 67μM of CB 1954 was reduced by the nitroreductase.
In contrast 50μM of 4-hydroxylamine was formed by the reduction
of 50μM of CB 1954 by the Walker NAD(P)H dehydrogenase
(quinone). Based on initial rates of 4-hydroxylamine formation
nitroreductase is 31.2 fold more active per mg protein than
35   NAD(P)H dehydrogenase (quinone) (or 62 fold more active by CB
1954 reduction) under the standard conditions used.

The rate of reduction of CB 1954 or product formation was the same when the co-factor was either NAD(P)H or NADH and when the reduction was performed under helium.

To show that the nitroreductase was producing a cytotoxic
5 species, the reduction of CB 1954 was carried out in the presence of V79 cells, which are insensitive to CB 1954. As shown in Table 4, a dramatic cytotoxic effect was observed in Hamster V79 cells - but only under those conditions in which the nitroreductase reduced CB 1954.

TABLE 4

The effect of CB 1954 on the survival of V79 cells in the presence of the *E. coli* B nitroreductase. All treatments were for 2 hours at 37°C and the cells were then plated out for their resulting colony-forming ablity. The nitroreductase concentration was 2μg/ml and NADH was

5   used as a cofactor.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
CONTROL	100	–
+ 500 μM NADH	100	–
+ 50 μM CB 1954	100	<1.0
+ NADH + CB 195	41	<1.0
+ Nitroreductase (NR)	94	–
+ NR + 50 μM CB 1954	99	<1.0
+ NR + CB 1954 + 500 μM NADH	0.024	72

10

EXAMPLE 3

Substrate specificity of the E. coli nitroreductase enzyme

The ability of the E. coli nitroreductase of the invention to
reduce nitro-compounds other than CB 1954 was determined by
5 HPLC by following the decrease in the peak area of NADH
resulting from its oxidation. The experiments were carried out
as above but the aliquots were injected onto a Partisphere SAX
(110 x 4.7 mm) HPLC column and eluted isocratically (1 ml/min)
with 75mM NaH_2PO_4. The results are shown in Table 5.

10 TABLE 5

The relative rates of reduction of various nitrobenzamides and
nitrobenzenes with E. coli nitroreductase enzyme. Reduction
rates were determined by the resulting oxidation of NADH. All
reactions were carried out at 37°C in air, with NADH (500μM) as
15 electron donor, at an initial substrate concentration of 100μM.

SUBSTRATE	RELATIVE RATE OF NADH OXIDATION
	NR
CB 1954	1.0
2,4-dinitro-5-(2-hydroxy-	
20 ethylamino)benzamide	0.04
2-amino-5-(aziridin-1-yl)-	
4-nitrobenzamide	<0.01
4-amino-5-(aziridin-1-yl)-	
4-nitrobenzamide	<0.01
25 5-chloro-2,4-dinitrobenzamide	22.4
3,5-dinitrobenzamide	75.5
2-nitrobenzamide	0.06
3-nitrobenzamide	1.8
4-nitrobenzamide	5.1
30 2,4-dinitrophenol	<0.01
5-nitro-2-furaldehydesemicarbazone	3.6
(nitrofurazone)	

EXAMPLE 4

Enzyme Kinetic and Inhibition Studies

Quinone reductase activities were assayed by a
spectrophotometric method using menadione as a substrate and
5   cytochrome c as a terminal electron acceptor as described in
Knox et al, Biochem. Pharmacol., 37, 4671-4677, 1988. Initial
rates of reaction were determined by linear regression analysis
(r>0.995) and kinetic parameters determined from the resulting
plots as described by Roberts et al, Biochem. Pharmacol. 38,
10 4137-4143, 1989. Protein concentration was determined using
the a conventional protein assay (Bio-Rad) calibrated against
bovine serum albumin.

Kinetic parameters for the E. coli nitroreductase and Walker
NAD(P)H, dehydrogenase (quinone) EC. 1.6.99.2, are given in
15 Table 6. Although both enzymes have comparable Km's for CB
1954, the Km of the nitroreductase for NADH is about 10 fold
less than the Walker enzyme. The absolute rates of reduction
of CB 1954 (i.e. under saturating conditions) by the two
enzymes is their k_{cat} values and this is 90 fold higher for the
20 E. coli nitroreductase. Menadione was also a substrate for
both enzymes with little difference in their respective k_{cat}'s
although the Km of nitroreductase for this substrate was 60
fold higher.

Dicoumarol was an inhibitor of the nitroreductase. No kinetic
25 parameters could be measured with respect to NADH. With
respect to menadione, dicoumarol was an uncompetitive inhibitor
with a Ki' of 1.90 ± 0.38μM.

TABLE 6

Kinetic parameters for the E. coli B nitroreductase and Walker NAD(P)H dehydrogenase (quinone).

		NR	WALKER
COMPOUND			
NADH	Km	~6μM	75μM
CB 1954	Km	862±145μM	826±46μM
	K_{cat}	360min^{-1}	4min^{-1}
MENADIONE	km	80μM	1.3μM
	K_{cat}	4.2x10^{4}min^{-1}	6.5x10^{4}min-1

EXAMPLE 5

The ability of the E. coli B nitroreductase to activate compounds other than CB 1954 to a cytotoxic species.

As shown in Table 7, a large cytotoxic effect was observed in human MAWI cells by the prodrugs CB 1837 and CB 10-107 but only under those conditions when the prodrug was reduced by the nitroreductase enzyme.

TABLE 7

The effect of enzyme-activated prodrugs on the survival of MAWI cells. 1 ml volumes of MAWI cells (2×10^5/ml) were incubated with 50 μM prodrug, 500 μM NADH, and 10 μg/ml the enzyme of
5 Example 1. After a 2 hour incubation at 37°C, the cells were harvested and assayed for their colony forming ability, and the supernatant assayed for the concentration of remaining prodrug by HPLC.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
10 CONTROL	100	-
+ 500μM NADH	100	-
+ 50μM CB 1837	97.4	<1.0
+ NADH + CB 1837	97.4	<1.0
+ 50μM CB 10-107	85.6	<1.0
15 + NADH + CB 10-107	87.9	<1.0
+ NR + CB 1837 + 500μM NADH	1.56	>95
+ NR + CB 10-107 + NADH	5.0	45

20 EXAMPLE 6
PREPARATION OF 1,4-DIHYDRO-NICOTINIC ACID RIBOSIDE FROM EITHER NICOTINIC ACID RIBOSIDE OR NICOTINIC ACID RIBOTIDE

(i) Preparation of nicotinic acid riboside from nicotinic acid ribotide.

25 A solution of nicotinic acid ribotide (nicotinic acid mononucleotide) (Sigma, Poole, U.K.) (25 mg) in aqueous buffer (tris 100 mM; pH 8.5; MgCl$_2$; 2.5 ml), was treated with 2000 units of alkaline phosphatase, type VII-S (100μl; 20,000 units/ml) (Sigma) at 37°C for 1 hour. The alkaline phosphatase
30 was separated from the digest by centrifugal molecular filtration (10,000 molecular weight limit; "Centricon 10"; Amicon, High Wycombe, U.K.). Dilutions (1:100) of the solution were analysed both before and after digestion with alkaline phosphatase by anion exchange high performance liquid
35 chromatography (Partisphere 5-SAX column, eluted isocratically

with 0.1M NaH$_2$PO$_4$, pH5, 1.5 ml/min, 10μl injection volume,
monitored by UV absorbance at 260 nm). The parent compound
eluted with a retention time of 1.18 minutes. Post digestion
examination indicated that a complete conversion had occurred

5 to give the novel title compound eluting with a retention time
of 0.63 minutes, taken to be indicative of dephosphorylation,
yielding the riboside.

(ii) Reduction of nicotinic acid riboside to 1,4-dihydro-
nicotinic acid riboside.

10 The method of Jarman and Searle (1972) was adopted. The method
was applied both to nicotinic acid riboside as produced above,
and also to chemically synthesised material (Jarman 1969).
Identical results were obtained with starting compound from
either source of origin.

15 To an aqueous solution of nicotinic acid riboside (5 ml; 4
mg/ml) was added 50 mg sodium carbonate, 50 mg sodium
bicarbonate, and 50 mg sodium hydrosulphite. The stoppered
solution was incubated at 37°C for 1 hour and the reduction
product separated by preparative reverse phase HPLC. The 5 ml

20 was injected onto a microsorb 5μm C18 (10 x 250 mm) reverse-
phase column (Rainin) and eluted by a gradient of methanol in
water (0-100% over 30 minutes) buffered at pH 5 by 10mM
NH$_4$CH$_3$COO at 4 ml/min. The eluate was continuously monitored
both by UV absorbance and by fluorescence and the reduction

25 product, (chromatographing with baseline resolution at a
retention time of 12-13 minutes), was characterised by both its
absorbance maximum at 326 nm (at pH5; 340 nm at pH7) and by
its fluorescence (Gilson 121 fluorometer with wide band glass
filters; excitation centred at 350 nm; emission at 450 nm),

30 neither of which properties are displayed by the parent
compound. Eluates of 4 ml. of a 2mM solution (assuming an ϵ_{340}
of 6200 (i.e. the same as NADH) were typical, indicating a
yield of 2.7 mg i.e. approximately 13%. Prior to experimental
usage, the preparations were analysed by analytical HPLC and

35 confirmed to be essentially pure.
References:-

(i) M. Jarman and F. Searle, Potential Coenzyme Inhibitors-V,
Biochem. Pharmacol. Vol 21, pp. 455-464, 1972.

(ii) M. Jarman, 4-Substituted Nicotinic acids and
Nicotinamides. Part III. Preparation of 4-Methylnicotinic
5 acid Riboside. J. Chem. Soc. (C), 918-920, 1969.

EXAMPLE 7

The ability of 1,4-dihydro-nicotinic acid riboside to act as a cofactor for the E. coli nitroreductase.

10 The ability of the nitroreductase enzyme to be able to use a
synthetic cofactor in place of NADH or NADPH for the reduction
of CB 1954 is shown in Figure 1. The nitroreductase enzyme can
use both 1,4-dihydro-nicotinic acid riboside and NADH with
equal efficiency as cofactors for the reduction of CB 1954. In
15 contrast Walker DT diaphorase cannot utilise this synthetic
cofactor. Therefore 1,4-dihydro-nicotinic acid riboside is a
selective cofactor for the E. coli nitroreductase enzyme and
cannot be used by mammalian DT diaphorase.

In the following Examples 8-12, "Silica gel" refers to
20 Merck silica gel 60, 70-230 mesh and Proton NMR spectra were
obtained at 300 MHz in $CDCl_3$. Temperatures are in °C.

EXAMPLE 8
4-[Bis(2-chloroethyl)amino]phenylcarbamic acid 4'-nitrobenzyl ester:

25 To a stirred solution of 4-nitrobenzyl chloroformate (295 mg)
in dry $CHCl_3$ (5 ml), a solution of 4-[bis(2-chloroethyl)amino]aniline hydrochloride (367 mg and NEt_3 (377
μl) in dry $CHCl_3$ (5 ml) was added over 5 min. After 1 hr the
solution was kept at 20° for 18 hr, then evaporated. The
30 residue was chromatographed on a column of silica gel with
$CHCl_3$ to give the title product as a yellow solid which
recrystallized from benzene/petroleum ether as prisms, m.p.
111-112°. Yield, 361 mg (64%). Chemical ionization mass

spectrum with CH$_4$: ion at m/z 412 (M + 1, relative intensity 1.00) indicates M = 411. C$_{18}$H$_{19}$N$_2$O$_4$Cl$_2$ requires M = 411 (for Cl^{35} isotope). NMR: δ3.57 (m, 4H, CH$_2$Cl or CH$_2$N), 3.69 (M, 4H, CH$_2$Cl or CH$_2$N), 5.28 (s, 2H, ArCH$_2$), 6.65 (d, 4H, ArH), 7.51 (d, 2H,
5 ArH) and 8.23 (d, 2H, ArH).

EXAMPLE 9
4-[Bis(2-chloroethyl)amino]phenyl 4'-nitrobenzyl carbonate

A solution of 4-nitrobenzyl chloroformate (58 mg) in dry CHCl$_3$ (1.5 ml) was added to a stirred, ice-cooled solution of 4-
10 [bis(2-chloroethyl)amino]-phenol hydrochloride (72 mg) and NEt$_3$ (74 μl) in dry CHCl$_3$ (2 ml). After 18 hr at 21°, the solution was evaporated and the residue chromatographed on a column of silica gel with CHCl$_3$/petroleum ether (3:2) to give the title compound which crystallized from ETOAc/petroleum ether as pale
15 yellow prisms, m.p. 77-79° (yield, 102 mg). FAB MS: ion at m/z 413 indicates M = 412 (C$_{18}$H$_{18}$N$_2$O$_5$Cl$_2$ requires M = 412). NMR (CDCl$_3$): δ 3.62 (m, NCH$_2$ or ClCH$_2$), 3.69 (m, NCH$_2$ or ClCH$_2$), 5.33 (s, ArCH$_2$), 6.64 (d, ArH), 7.05 (d, ArH), 7.59 (d, ArH) and 8.25 (d, ArH).

20 EXAMPLE 10
N-4-Nitrobenzyloxycarbonyl-actinomycin D:

Actinomycin D (AMD, 41 mg) in MeOH (5 ml) was hydrogenated over 10% Pd/C for 2 hr, then evaporated in vacuo. The residue under N$_2$ was dissolved in a solution of 4-nitrobenzyl chloroformate
25 (18 mg) in dry CHCl$_3$ (1.5 ml) and a solution of NEt$_3$ (10 μl) in CHCl$_3$ (1.5 ml) was then added. After stirring under N$_2$ for 24 hr, the catalyst was filtered off and the solution, after dilution with MeOH (200 ml), was aerated for 3 days. The solution was evaporated and the product was purified to remove
30 AMD by semi-preparative HPLC twice on a 1 cm diameter column of reversed-phased C$_{18}$-bonded silica with a 50-100% gradient of MeCN in H$_2$O. The title compound was crystallised from ethyl acetate/petroleum ether as red prisms. Yield, 31 mg (66%). Electrospray mass spectrum: ions at m/z 1434.5 (M
35 + H) and 1456.4 (M + Na) indicates M = 1433.5. C$_{70}$H$_{91}$N$_{13}$O$_{20}$

(lowest mass isotope) requires M = 1433.65. NMR gave the same
signals as AMD plus δ6.71 (d, 1H, ArCH$_2$), 7.09 (d, 1H, ArCH$_2$)
and additional signals in the aromatic region.

EXAMPLE 11

5   N-4-nitrobenzyloxycarbonyl-doxorubicin VI

Doxorubicin hydrochloride (2.25 mg) was dissolved in
dimethylformamide (DMF) (0.3 ml) containing triethylamine
(NEt$_3$) (0.55 μl) and a solution of 4-nitrobenzyl-4'-
nitrophenylcarbonate (1.4 ml) in DMF (0.1 ml) was added. After
10   stirring in the dark for 3 days, the mixture was separated by
HPLC on a column (1 cm. diam.) of C$_{18}$ reversed-phase silica with
a gradient of 25 to 100% MeCN in 0.01M formate buffer (pH 4.0).
The principal red fraction was concentrated and
rechromatographed with H$_2$O in place of the formate buffer.
15   Evaporation in vacuo afforded the product (2.1 mg) as an
amorphous red powder. Electrospray MS: ion at 723 (M+H)
indicates M = 722. C$_{35}$H$_{34}$N$_2$O$_{15}$ requires M = 722.

EXAMPLE 12

N-4-Nitrobenzyloxycarbonyl-mitomycin C

20   A solution of mitomycin C (36 mg) in dimethyl formamide (DMF)
(2 ml) containing NEt$_3$ 14 μl) was added to 4-nitrobenzyl
chloroformate (30 mg) and the mixture was stirred at 21° for 4
hr. After evaporation in vacuo, the residue was
chromatographed on a column of silica gel with EtOAc to give
25   the title compound as a dark red solid (49 mg). Electrospray
MS: ion at 514 (M+H) indicates M = 513. C$_{23}$H$_{23}$N$_5$O$_9$ requires M =
513.

EXAMPLE 13

Formation of actinomycin D by the action of the nitroreductase
30   upon prodrug V:

V (100 μM) and cofactor (500 μM NADH) were incubated with
enzyme (2 μg/ml E. coli B nitroreductase of Example 1 in 10 mM
sodium phosphate buffer (pH7) in air at 37°C. At various

times, aliquots (20 μl) were injected onto a Microsorb C18
reverse-phase (240 x 4.7 mm) HPLC column and eluted
isocratically (1 ml/min) with 80% acetonitrile in water.   The
eluate was continuously monitored for absorption at 280 nm and
5 the concentration of drug calculated by integration of the peak
corresponding to this compound on the HPLC trace.   Only when
the enzyme was present was actinomycin D released from the
prodrug.

Disappearance of the prodrug N-4-nitro-benzyloxycarbonyl-
10 actinomycin D (V) and formation of actinomycin D during
incubation with the E. coli B nitroreductase of Example 1 is
shown in Figure 2.

EXAMPLE 14
The formation of mitomycin C by the action of the
15 nitroreductase enzyme upon prodrug VII

Prodrug VII (100μM) and cofactor (500μM NADH) were incubated
with enzyme (5μg/ml E. coli B nitroreductase of Example 1) in
10mM sodium phosphate buffer (pH7) in air at 37°C.   At various
times aliquots (10μl) were injected onto a Partisphere C18
20 reverse-phase (150x4.7mm) HPLC column and eluted isocratically
(2.0ml/min) with 50% methanol in water.   The eluate was
continuously monitored for absorption at 260 and 340 nm and the
concentration of the drugs calculated by integration of the
peak corresponding to the compound on the HPLC trace.

25 Disappearance of prodrug VII and the formation of mitomycin C
during this incubation is shown in Figure 3.   Only in the
presence of the enzyme is mitomycin C released from the
prodrug.

EXAMPLE 15
30 Activation of prodrug I where R^1NH_2 = III and prodrug V by the
nitroreductase:

Generation of cytotoxicity by the action of the
E. coli nitroreductase of Example 1 upon the prodrugs

4-[bis(2-chloroethyl)amino]phenylcarbamic acid
4'-nitrobenzyl ester (I where R'NH₂ = III) and N-4-
nitrobenzyloxy-carbonyl-actinomycin D (V) are shown in Table
8.

5   TABLE 8

The effect of enzyme-activated prodrugs on the survival of V79
cells.   1 ml volumes of V79 cells (2×10^5/ml) were incubated
with prodrug, 500 μM NADH, and 10 μg/ml enzyme. After a 2 hour
incubation at 37°C, the cells were harvested and assayed for
10 their colony forming ability, and the supernatant assayed for
the concentration of remaining prodrug by HPLC.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
CONTROL	100	-
+ 500μM NADH	100	-
+ 50μM (I where R'NH₂=III)	27.1	<1.0
+ NR + 50μM (I where R'NH₂=III) + 500μM NADH	.0.001	30
+ 1μM V	98.3	<1.0
+ 10μM V	39.3	<1.0
+ NR + 1μM V + 500 μM NADH	27.4	90.5
+ NR + 10μM V + 500μM NADH	.0.0001	93
+ NR + CB 10-107 + NADH	5.0	45

25 EXAMPLE 16
Preparation and binding of Antibody: Enzyme Conjugate

Antibody:enzyme conjugate of A5B7 F(ab)₂: nitroreductase was
prepared using the heterobifuncitional agents succinimidyl 4-
(p-maleimidophenyl)butyrate (SMPB) to insert active maleimide
30 groups into the immunoglobulin, and 2-mercapto-[S-acetyl]acetic
acid N-hydroxysuccinimide (SATA) to thiolate the E. coli
nitroreductase.   On mixing the proteins the maleimide groups
react with thiols to form a thioether bond.   The molar ratio of
SMPB:immunoglobulin used was 10:1 and the molar ratio of

SATA:nitroreductase was 4:1. Antibody:enzyme conjugate thus prepared was isolated from high molecular weight aggregates and uncoupled components by gel filtration chromatography using a calibrated column (16 x 700mm) of Superdex G200. The column
5 was equilibrated in phosphate buffered saline (PBS) and eluted with the same buffer at a flow rate of 1.0ml/min. Fractions containing material corresponding in molecular weight to 2:1 and 1:1 conjugate (316KDa and 233KDa respectively) were pooled and rechromatographed on the same column and samples from
10 pooled fractions were run on 4-15% SDS-PAGE gels (Pharmacia Phastgels, run for 60vh) under non-reducing conditions together with calibration proteins. The conjugate was present as material with Mr 125 KDa (corresponding to 1:1 F(ab')$_2$:nitroreductase and higher molecular weight conjugates
15 together with less amounts of free F(ab')$_2$ and nitroreductase.

The enzymic activity of the conjugate was established by the routine assay using CB1954 as substrate. The conjugate was shown to bind to plates coated with 1µg/ml CEA antigen and to contain binding sites for both rabbit anti-mouse immunoglobulin
20 and rabbit anti-nitroreductase secondary antibodies (Figure 4). Samples of uncoupled F(ab')$_2$ and nitroreductase were used to confirm the specificity of the secondary antibody binding.

The antibody binding was determined using a standard horse radish peroxidase (HRP) colorimetric ELISA assay, with the
25 results being read at 405nm. The inverted open triangles on Figure 4 show that bound A5B7 F(ab')$_2$-nitroreductase conjugate can be detected with a goat anti-mouse immunoglobulin antibody, and the closed inverted triangles show that the conjugate is also detected by a rabbit anti-NR antibody (the anti-NR
30 antibody being detected via the use of a goat anti-rabbit immunoglobulin antibody. The controls shown in Figure 4 are: closed circles = binding of unconjugated A5B7 F(ab')$_2$; open squares = A5B7 F(ab')$_2$-NR conjugate detected with goat anti-rabbit immunoglobulin; closed squares = NR only detected with
35 rabbit anti-NR; open triangles = NR only detected with goat anti-mouse immunoglobulin.

EXAMPLE 17

In vivo toxicity of prodrug.

The actinomycin D prodrug (AMDPD) of formula V was tested for
toxicity in mice. Groups of 3 mice were given 1, 10 or 100
5 mg/kg body weight i.p. of AMPD, and two further groups of 3
mice were given 1 and 10 mg/kg body weight i.p. of actinomycin
D (AMD) dissolved in arachis oil. A further group of 3 mice
were untreated, and a final group of 3 mice were given arachis
oil i.p. only.

10 The body weight of the mice were monitored over 9 days. The
results are shown on Table 9. All mice treated with 10mg/kg of
AMD were dead by day 1. A similar result was obtained with a
dose of 5mg/kg. These data indicate that AMDPD is at least 20
to 100 fold less toxic than AMD. In practice, the toxicity of
15 AMDPD is likely to be even lower, since the preparation of
AMDPD used contains about 1% unconverted AMD.

TABLE 9

Toxicity of AMD and AMDPD

Compound	Dose	Weight as % of Day 0			
		Day 2	Day 3	Day 7	Day 9
Prodrug	100	96.4 (1 dead)	95.6	95	96
	10	109	105	108	110
	1.0	102	101	101	103
Active Drug	10	All dead on day 1.			
	1.0	98.6	95	101.6	103
Control	–	104	103	106	106
Oil		99	99	101	102

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Cancer Research Campaign Technology Limited
        (B) STREET: Cambridge House, 6-10 Cambridge Terrace
               Regent's Park
        (C) CITY: LONDON, GB
        (F) POSTAL CODE: NW1 4JL

    (ii) TITLE OF INVENTION: Improvements Relating to Drug
                  Delivery Systems

    (iii) NUMBER OF SEQUENCES: 2

    (iv) COMPUTER READABLE FORM:
        Not Applicable

    (v) CURRENT APPLICATION DATA:
        APPLICATION NUMBER: PCT/GB92/.....

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1167 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Escherichia coli

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 176..829

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TCGCGATCTG ATCAACGATT CGTGGAATCT GGTGGTTGAT GGTCTGGCTA AACGCGATCA      60

AAAAAGAGTG CGTCCAGGCT AAAGCGGAAA TCTATAGCGC ATTTTTCTCG CTTACCATTT     120

CTCGTTGAAC CTTGTAATCT GCTGGCACGC AAAATTACTT TCACATGGAG TCTTT ATG     178
                                                             Met
                                                               1

GAT ATC ATT TCT GTC GCC TTA AAG CGT CAT TCC ACT AAG GCA TTT GAT     226
Asp Ile Ile Ser Val Ala Leu Lys Arg His Ser Thr Lys Ala Phe Asp
          5                   10                  15

GCC AGC AAA AAA CTT ACC CCG GAA CAG GCC GAG CAG ATC AAA ACG CTA     274
Ala Ser Lys Lys Leu Thr Pro Glu Gln Ala Glu Gln Ile Lys Thr Leu
      20                  25                  30
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CTG CAA TAC AGC CCA TCC AGC ACC AAC TCC CAG CCG TGG CAT TTT ATT      322
Leu Gln Tyr Ser Pro Ser Ser Thr Asn Ser Gln Pro Trp His Phe Ile
    35              40                  45

GTT GCC AGC ACG GAA GAA GGT AAA GCG CGT GTT GCC AAA TCC GCT GCC      370
Val Ala Ser Thr Glu Glu Gly Lys Ala Arg Val Ala Lys Ser Ala Ala
50              55                  60                  65

GGT AAT TAC GTG TTC AAC GAG CGT AAA ATG CTT GAT GCC TCG CAC GTC      418
Gly Asn Tyr Val Phe Asn Glu Arg Lys Met Leu Asp Ala Ser His Val
            70                  75                  80

GTG GTG TTC TGT GCA AAA ACC GCG ATG GAC GAT GTC TGG CTG AAG CTG      466
Val Val Phe Cys Ala Lys Thr Ala Met Asp Asp Val Trp Leu Lys Leu
            85                  90                  95

GTT GTT GAC CAG GAA GAT GCC GAT GGC CGC TTT GCC ACG CCG GAA GCG      514
Val Val Asp Gln Glu Asp Ala Asp Gly Arg Phe Ala Thr Pro Glu Ala
            100                 105                 110

AAA GCC GCG AAC GAT AAA GGT CGC AAG TTC TTC GCT GAT ATG CAC CGT      562
Lys Ala Ala Asn Asp Lys Gly Arg Lys Phe Phe Ala Asp Met His Arg
        115                 120                 125

AAA GAT CTG CAT GAT GAT GCA GAG TGG ATG GCA AAA CAG GTT TAT CTC      610
Lys Asp Leu His Asp Asp Ala Glu Trp Met Ala Lys Gln Val Tyr Leu
130             135                 140                 145

AAC GTC GGT AAC TTC CTG CTC GGC GTG GCG GCT CTG GGT CTG GAC GCG      658
Asn Val Gly Asn Phe Leu Leu Gly Val Ala Ala Leu Gly Leu Asp Ala
            150                 155                 160

GTA CCC ATC GAA GGT TTT GAC GCC GCC ATC CTC GAT GCA GAA TTT GGT      706
Val Pro Ile Glu Gly Phe Asp Ala Ala Ile Leu Asp Ala Glu Phe Gly
            165                 170                 175

CTG AAA GAG AAA GGC TAC ACC AGT CTG GTG GTT GTT CCG GTA GGT CAT      754
Leu Lys Glu Lys Gly Tyr Thr Ser Leu Val Val Val Pro Val Gly His
        180                 185                 190

CAC AGC GTT GAA GAT TTT AAC GCT ACG CTG CCG AAA TCT CGT CTG CCG      802
His Ser Val Glu Asp Phe Asn Ala Thr Leu Pro Lys Ser Arg Leu Pro
    195                 200                 205

CAA AAC ATC ACC TTA ACC GAA GTG TAATTCTCTC TTGCCGGGCA TCTGCCCGGC     856
Gln Asn Ile Thr Leu Thr Glu Val
210                 215

TATTTCCTCT CAGATTCTCC TGATTTGCAT AACCCTGTTT CAGCCGTCAT CATAGGCTGC     916

TGTTGTATAA AGGAGACGTT ATGCAGGATT TAATATCCCA GGTTGAAGAT TTAGCGGGTA     976

TTGAGATCGA TCACACCACC TCGATGGTGA TGATTTTCGG TATTATTTTT CTGACCGCCG    1036

TCGTGGTGCA TATTATTTTG CATTGGGTGG TACTGCGGAC CTTCGAAAAA CGTGCCATCG    1096

CCAGTTCACG GCTTTGGTTG CAAATCATTA CCCAGAATAA ACTCTTCCAC CGTTTAGCTT    1156
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TTACCCTGCA G   1167

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 217 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asp Ile Ile Ser Val Ala Leu Lys Arg His Ser Thr Lys Ala Phe
  1           5               10              15

Asp Ala Ser Lys Lys Leu Thr Pro Glu Gln Ala Glu Gln Ile Lys Thr
         20             25              30

Leu Leu Gln Tyr Ser Pro Ser Ser Thr Asn Ser Gln Pro Trp His Phe
         35             40              45

Ile Val Ala Ser Thr Glu Glu Gly Lys Ala Arg Val Ala Lys Ser Ala
         50             55              60

Ala Gly Asn Tyr Val Phe Asn Glu Arg Lys Met Leu Asp Ala Ser His
 65             70             75              80

Val Val Val Phe Cys Ala Lys Thr Ala Met Asp Asp Val Trp Leu Lys
                85             90              95

Leu Val Val Asp Gln Glu Asp Ala Asp Gly Arg Phe Ala Thr Pro Glu
            100            105             110

Ala Lys Ala Ala Asn Asp Lys Gly Arg Lys Phe Phe Ala Asp Met His
         115            120            125

Arg Lys Asp Leu His Asp Asp Ala Glu Trp Met Ala Lys Gln Val Tyr
         130            135            140

Leu Asn Val Gly Asn Phe Leu Leu Gly Val Ala Ala Leu Gly Leu Asp
145            150            155            160

Ala Val Pro Ile Glu Gly Phe Asp Ala Ala Ile Leu Asp Ala Glu Phe
            165            170            175

Gly Leu Lys Glu Lys Gly Tyr Thr Ser Leu Val Val Val Pro Val Gly
         180            185            190

His His Ser Val Glu Asp Phe Asn Ala Thr Leu Pro Lys Ser Arg Leu
         195            200            205

Pro Gln Asn Ile Thr Leu Thr Glu Val
210            215
```

CLAIMS

1.   A nitroreductase, obtainable from a bacterium having the following characteristics:

    1.   It is a flavoprotein having a molecular weight in the range 20-60 Kilodaltons;

    2.   It requires either NADH or NAD(P)H or analogues thereof as a cofactor;

    3.   It has a Km for NADH or NAD(P)H in the range 1-100μM; and

    4.   It is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form e.g. the hydroxylamine.

2.   A nitroreductase according to claim 1 obtainable from the cells of E. coli B , E. coli C, Thermus aquaticus, Bacillus amyloliquifaciens or Bacillus caldotenax.

3.   A nitroreductase having the amino acid sequence of Seq. ID No. 2.

4.   A DNA encoding for the nitroreductase of claim 3.

5.   A DNA according to claim 4 which comprises the residues 176 to 829 of the DNA of Seq. ID No. 1.

6.   An antibody capable of binding a nitroreductase according to any one of claims 1 to 3.

7.   An antibody according to claim 6 which is a natural or recombinant monoclonal antibody or fragment thereof.

8.   A conjugate of (i) a targeting agent for a tumour and (ii) a nitroreductase according to any one of claims 1 to 3, optionally in association with a pharmaceutically acceptable carrier or diluent.

9.   A conjugate according to claim 8 wherein the targeting agent is a monoclonal antibody which will bind a tumour-

associated antigen.

10.   A compound of the forumula (I) or (II):
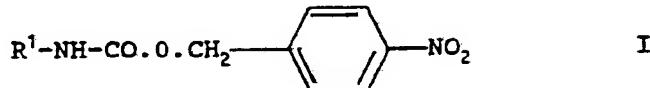
$$R^1\text{-NH-CO.O.CH}_2\text{---}\bigcirc\text{---NO}_2 \qquad \text{I}$$

or:

$$R^2\text{-O.CO.O.CH}_2\text{---}\bigcirc\text{---NO}_2 \qquad \text{II}$$

where R^1 and R^2 are groups such that the compound R^1NH_2 and R^2OH are cytotoxic compounds.

11.   A compound according to claim 10 wherein the compound R^1NH_2 is a nitrogen mustard compound.

12.   A compound according to claim 11 of the formula (IV):

$$(ClCH_2CH_2)_2N\text{---}\underset{R'\quad R''}{\bigcirc}\text{---NH}_2 \qquad \text{IV}$$
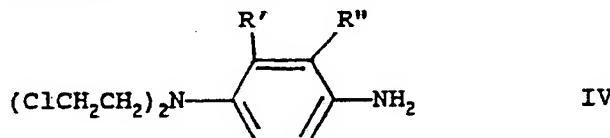
where R' and R'' are H, F or CH_3.

13.   A compound according to claim 12 where R' and R'' are both hydrogen.

14.   A compound according to claim 10 which is a phenolic nitrogen mustard.

15.   A compound according to claim 14 of the formula (VIII):

$$(ClCH_2CH_2)_2N\text{---}\bigcirc\text{---O.CO.O.CH}_2\text{---}\bigcirc\text{---NO}_2 \qquad \text{VIII}$$

16.   A compound of the formula (V), (VI), (VII):

- 43 -

V
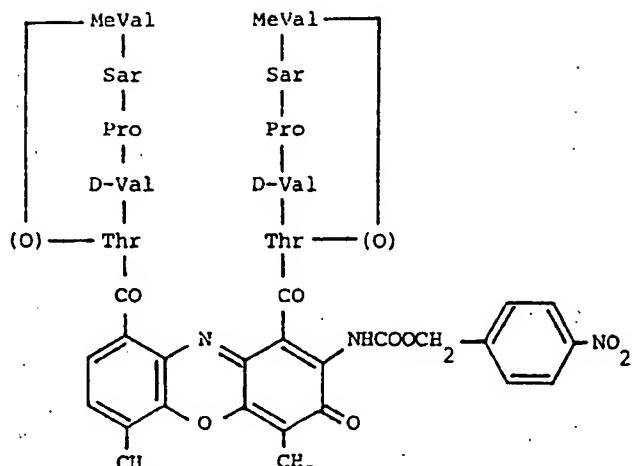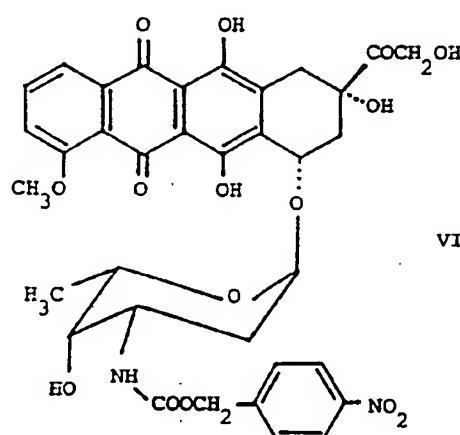
VI

VII
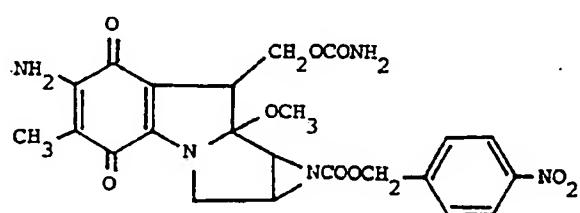
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17. A pharmaceutical compostition comprising a compound according to any one of claims 9 to 16 in association with a pharmaceutically acceptable carrier or diluent.

18. A compound of the formula (IX):

IX
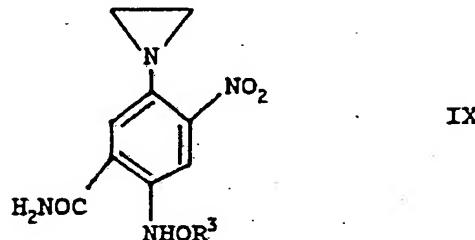
wherein R^3 is H, an acyl group or a hydrocarbyl group containing up to 6 carbon atoms.

19. A compound of the formula (X):
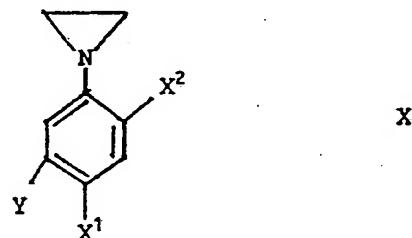
X

wherein X^1 and X^2, which may be the same or different, are each $NHOR^5$ or NO_2 with the proviso that X^1 and X^2 are not both NO_2, where R^5 is H or a carboxylic acyl or hydrocarbyl group and Y is H or $CON(CH_3)_2$.

20. A pharmaceutical composition comprising a compound according to claim 18 or 19 in association with a pharmaceutically acceptable carrier or diluent.

21. A system comprising (i) a conjugate according to claim 8 or 9 and (ii) a prodrug according to any one of claims 10 to 16; or a composition according to claim 17; or a nitro-precursor of a compound according to claims 18 or 19 or a

pharmaceutical composition comprising such a compound in
association with a pharmaceutically acceptable carrier or
diluent.

22.   A system according to claim 21 for use in a method of
treatment of the human or animal body.

23.   The riboside of 1,4-dihydro-nicotinic acid, optionally in
association with a pharmaceutically acceptable carrier or
diluent.

24.   A system according to claim 21 which further comprises
(iii) the riboside according to claim 23 .

25.   A system according to claim 24 for use in a method of
treatment of the human or animal body.

26.   A process for the production of a nitroreductase, having
the following characteristics:
       1.   It is a flavoprotein having a molecular weight in
the range 20-60 Kilodaltons;
       2.   It requires either NADH or NAD(P)H or analogues
thereof as a cofactor;
       3.   It has a Km for NADH or NAD(P)H in the range
1-100μM; and
       4.   It is capable of reducing either or both nitro
groups of CB 1954 and analogues thereof to a cytotoxic form
e.g. the hydroxylamine, which comprises disrupting a bacterial
cell containing the nitroreductase and subjecting the cell
contents to chromatographic separation and isolating the
nitroreductase.

27.   A process according to claim 26 wherein the cells are E.
coli B , E. coli C, Thermus aquaticus, Bacillus
amyloliquifaciens or Bacillus caldotenax.

28.   A process for the production of a nitroreductase having
the amino acid sequence of Seq. ID No. 2 which comprises
expressing DNA encoding the nitroreductase in an

expression vector contained in a host cell, and recovering the nitroreductase.

29. A process for the production of a prodrug of formula (I) or (II) as defined in claim 10 or a prodrug of formula (V), (VI) or (VII) as defined in claim 16 which comprises reacting the corresponding drug precursor with 4-nitrobenzyl chloroformate under anhydrous conditions.

30. A process for making a compound of the formula (IX) according to claim 18 or a compound of formula (X) according to claim 19 which comprises subjecting the corresponding dinitro compound to the action of a nitroreductase according to any one of claims 1 to 3.

31. A process for making the riboside of 1,4-dihydro-nicotinic acid which comprises reducing nicotinic acid riboside.

32. A process for making a conjugate of a targeting agent for a tumour and an enzyme as defined in any one of claims 1 to 3 which comprised covalently linking the agent to the enzyme using a covalent linker.

33. A process according to claim 33 wherein the targeting agent is a monoclonal antibody.

34. A method of treatment of neoplasia in a human or animal host requring such treatment which comprises administering to the host an effective amount of (i) a nitroreductase according to claim 1 or 3 conjugated with a targeting agent for a tumour; and (ii) a p-nitrobenzyloxycarbonyl compound of Formula I or II as defined in claim 10 or a compound of formula V, VI or VII as defined in claim 16 which is a prodrug for an anti-tumour agent

35. A method according to claim 34 wherein components (i) and (ii) are administered sequentially.

36. A method according to claim 34 which further comprises the administration of (iii) a ribotide or riboside of nicotinic acid or nicotinamide as cofactor for the enzyme.

37. A method for the treatment of neoplasia in a human or animal host requiring such treatment which comprises administering to the host (i) a nitroreductase according to claim 1 or 3 conjugated with a targeting agent for a tumour and (ii) an effective amount of a nitro compound which is a prodrug for an anti-tumour agents of the formula IX or X as defined in claims 18 or 19.

38. A method according to claim 36 which further comprises the administration of (iii) a ribotide or riboside of nicotinic acid or nicotinamide as cofactor for the enzyme.

Fig.1.
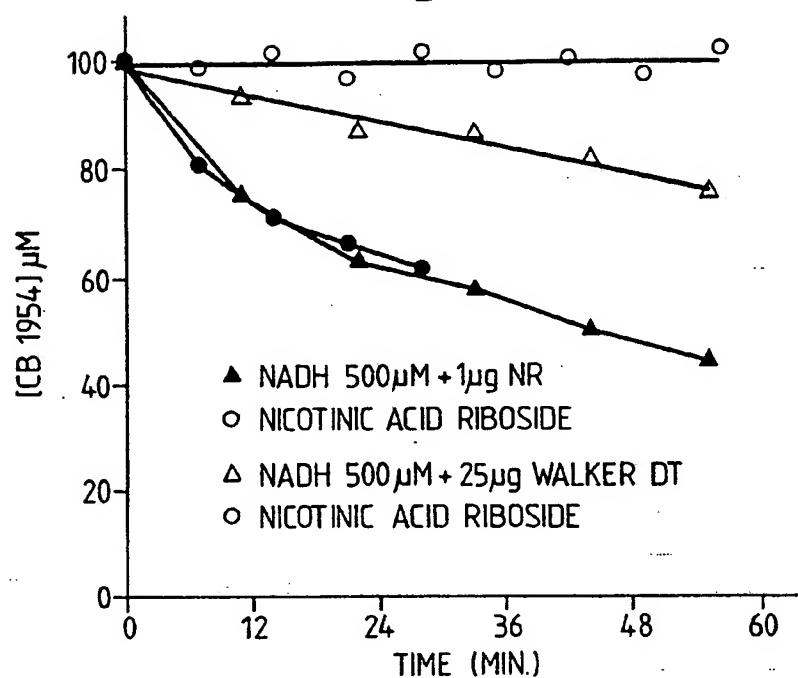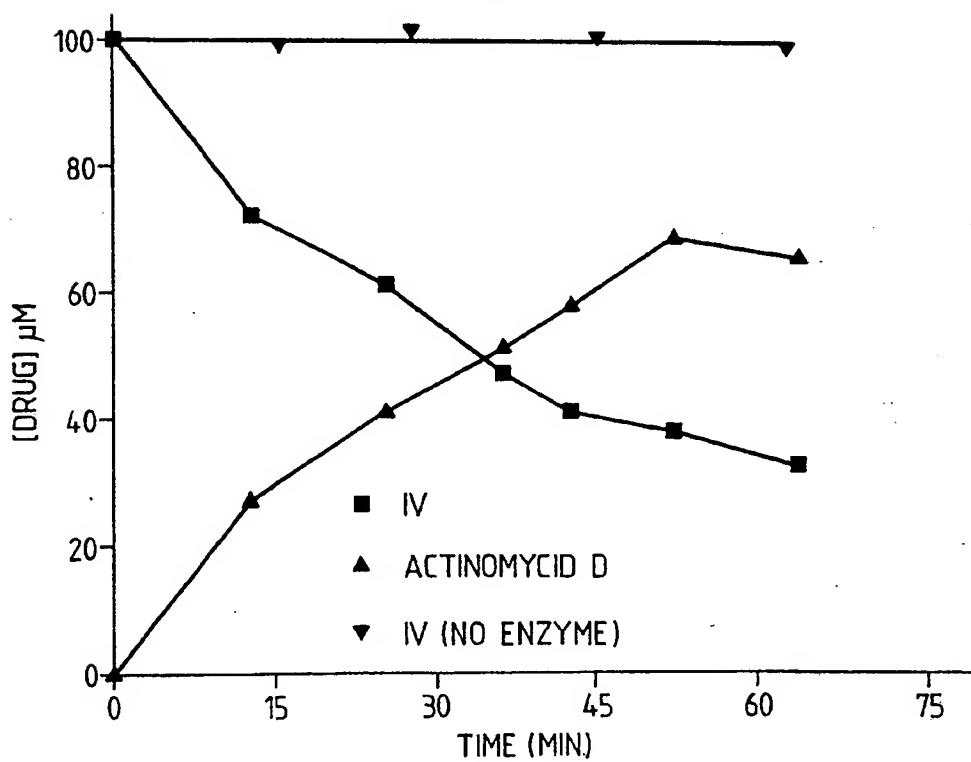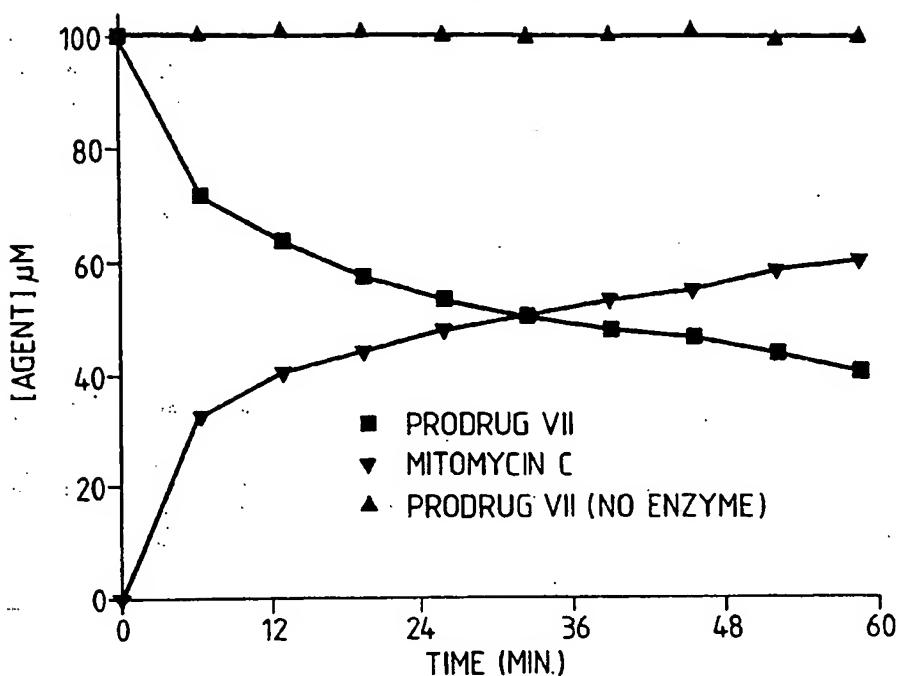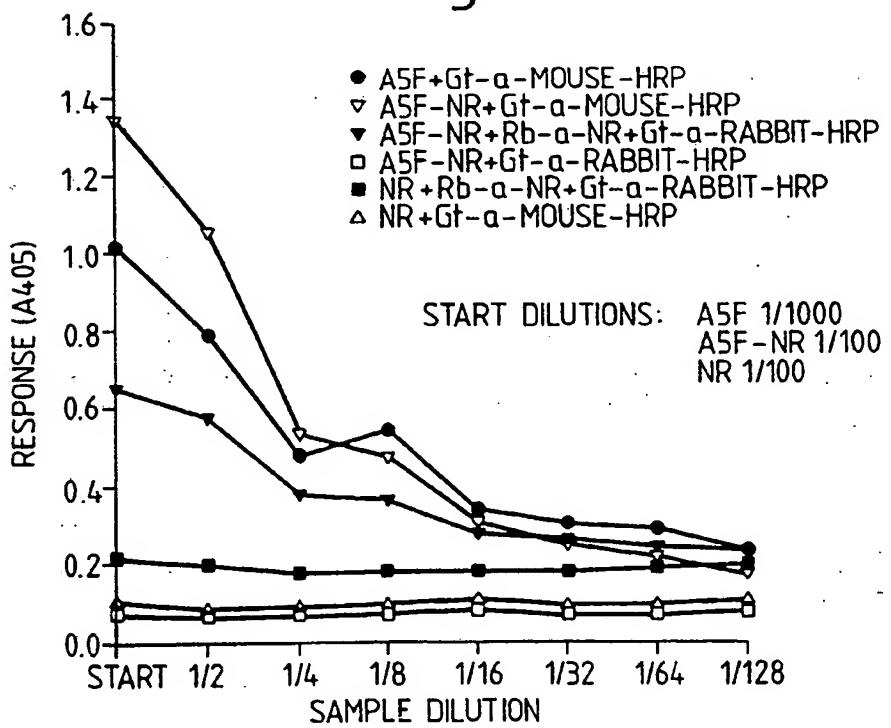
▲ NADH 500μM + 1μg NR
○ NICOTINIC ACID RIBOSIDE
△ NADH 500μM + 25μg WALKER DT
○ NICOTINIC ACID RIBOSIDE

[CB 1954] μM

TIME (MIN.)

Fig.2.

■ IV
▲ ACTINOMYCID D
▼ IV (NO ENZYME)

[DRUG] μM

TIME (MIN.)

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Fig.3.

Plot of [AGENT] µM versus TIME (MIN.) from 0 to 60.

- ■ PRODRUG VII
- ▼ MITOMYCIN C
- ▲ PRODRUG VII (NO ENZYME)

Fig.4.

Plot of RESPONSE (A405) versus SAMPLE DILUTION.

- ● A5F+Gt-a-MOUSE-HRP
- ▽ A5F-NR+Gt-a-MOUSE-HRP
- ▼ A5F-NR+Rb-a-NR+Gt-a-RABBIT-HRP
- □ A5F-NR+Gt-a-RABBIT-HRP
- ■ NR+Rb-a-NR+Gt-a-RABBIT-HRP
- △ NR+Gt-a-MOUSE-HRP

START DILUTIONS: A5F 1/1000
A5F-NR 1/100
NR 1/100

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L CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.Cl. 5 C12N15/53;	C12N9/02;	C12P21/08;	C12P7/10
C07K7/06;	C07D487/14;	C07D203/14;	C07C271/18

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; C07K ; C07D C07C ; C07H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOCHEMICAL PHARMACOLOGY vol. 37, no. 24, 1988, pages 4661 - 4669 RICHARD J. KNOX ET AL. 'A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1594) by a nitroreductase enzyme in Walker carcinoma cells' see figure 1	18
A	see page 4664, right column, paragraph 1 -paragraph 2 see page 4667, left column, paragraph 1 - right column, paragraph 2 see page 4668, right column, paragraph 3	1,8,9, 21,22,26

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° Special categories of cited documents :[10]	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JANUARY 1993	
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 330 432 (ROBERTS, JOHN J. ET AL.) 30 August 1989 see column 1, line 18 - column 2, line 15 see column 2, line 38 - column 4, line 33 ---	1,8, 18-22, 26,30,37
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 7, 5 March 1991, BALTIMORE US pages 4126 - 4130 CHRISTOPHER BRYANT ET AL. 'Cloning, nucleotide sequence, and expression of the nitroreductase gene from Enterobacter cloacae' cited in the application see page 4126, left column, paragraph 1 see page 4128, left column, last paragraph - page 4129, right column, paragraph 1; figure 4 see page 4130, left column, last paragraph ---	1-5,28
A	EP,A,0 317 956 (BRISTOL-MYERS COMPANY) 31 May 1989 see page 2, line 37 - line 41; figure 3 ---	16
A	EP,A,0 441 218 (BEHRINGWERKE) 14 August 1991 see abstract ---	16
A	US,A,4 680 382 (SISIR K. SENGUPTA). 14 July 1987 see column 1, line 5 - line 43 ---	16
A	CHEMICAL ABSTRACTS, vol. 95, 1981, Columbus, Ohio, US; abstract no. 98201a, MIKHAILOPULO, I.A. ET AL. 'Synthesis of glycosides of nicotinamide and nicotinamide mononucleotide' page 700 ;column L ; see abstract & SYNTHESIS vol. 5, 1981, STUTTGART DE pages 388 - 389 -----	23

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X]   Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 34-38 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ]   Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ]   Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ]   As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ]   As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ]   As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ]   No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ]   The additional search fees were accompanied by the applicant's protest.

                              [ ]   No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9201947
SA 65801

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0330432	30-08-89	AU-A- EP-A- WO-A- JP-T-	3439889 0429454 8907592 3503761	06-09-89 05-06-91 24-08-89 22-08-91
EP-A-0317956	31-05-89	AU-A- JP-A-	2582688 1165586	25-05-89 29-06-89
EP-A-0441218	14-08-91	DE-A- AU-A-	4002888 7011791	08-08-91 08-08-91
US-A-4680382	14-07-87	None		

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